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14. ABSTRACT

microRNAs (miRNAs) are small, regulatory RNAs that silence target genes by repressing translation and destabilizing mRNAs (1). Recent studies have demonstrated the importance of miRNAs in molecular mechanisms for the oncogenic and tumor suppressor pathways (2) (3) (4). We have identified a novel oncogenic miRNA polycistron, mir17-92, as a potential human oncogene in B-cell lymphomas (5). Since mir17-92 is overexpressed in a subset of breast tumor samples, we explored the role of mir17-92 in the tumorigenesis of breast tumor. Mir-17-92 is able to collaborate with c-myc to accelerate breast tumor formation, and we are further characterizing the molecular mechanisms for this oncogenic collaboration. In addition, we have recently identified a miRNA component of the p53 pathway. mir-34 family microRNAs are direct transcriptional target of p53, and are able to induce growth arrest in both primary cells and tumor cell lines. Since p53 pathway is an important tumor suppressor mechanism in breast cancer development, we will further look into the roles of mir-34 miRNAs in preventing tumorigenesis in breast epithelia.

15. SUBJECT TERMS
microRNA, breast cancer, non-coding RNA

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Table of Contents

Page

INTRODUCTION

The broad goal of this project is to explore the role of microRNAs (miRNAs) in tumorigenesis, and to apply these findings to the discovery of new therapeutic targets for breast cancer. Specifically, we have continued our characterization of mir17-92 in its oncogenic effects in breast tumor formation. In addition, we identified a novel miRNA family, *mir-34*, as an important component of the p53 pathway. Since the p53 pathway is an important tumor suppressor pathway regulating the tumorigenesis of breast cancer, this finding elucidated a novel molecular mechanism underlying the disease mechanism, and provide new therapeutical insights.

A global reduction in microRNA (miRNA) levels is often observed in human cancers, suggesting that small RNAs play an intrinsic role in tumor suppression. To identify miRNA components of tumor suppressor pathways, we compared miRNA expression profiles of wild type and p53 deficient mouse embryonic fibroblasts. This analysis revealed a family of miRNAs, *mir-34*a-c, whose expression reflected p53 status. All three *mir-34* family genes are direct transcriptional targets of p53, whose induction by DNA damage and oncogene stress depends on p53 both *in vitro* and *in vivo*. Ectopic expression of *mir-34* induces cell cycle arrest in both primary and tumor-derived cell lines, consistent with the ability of *mir-34* to down-regulate genes promoting DNA replication and cell cycle progression. The p53 network suppresses tumor formation though coordinated activation of multiple targets, and *mir-34* may act in concert with other effectors to promote growth arrest in response to p53 activation.

BODY

Overexpression of mir17-92 in mammary reconstitution model

The biological roles of many oncogenes have been revealed through the study of mouse models. Our goal is to determine whether mir-17-92 contributes to breast tumorigenesis, and to elucidate the likely mechanisms. This would be achieved by creating mosaic mammary glands in which a subset of cells have enforced mir17-92 expression. In collaboration with Dr. Senthil Muthuswamy (CSHL), we have developed a mammary fat pad transplantation system to test the oncogenic functions of mir-17-92 in breast tumorigenesis (Fig1). Immortalized mammary epithelia cell line, Comma-D cells, are infected with c-myc oncogene and a MSCV retroviral vectors overexpressing mir-17-19b miRNA cluster, a subcluster of mir17-92, before transplanted into a cleared mammary fat pad of three-week old mice. Overexpression of mir-17-19b collaborated with c-myc and accerlated the tumorigenesis of breast cancer in this mouse model (Fig1).

Fig1. Overexpression of *mir-17-19b* accelerate the tumorigenesis of breast tumor.

mir-34s are identified as p53 transcriptional targets

A global reduction in microRNA (miRNA) levels is often observed in human cancers, suggesting that small RNAs play an intrinsic role in tumor suppression. To explore further whether miRNAs constitute part of intrinsic tumor suppressor mechanisms, we examined the miRNA expression profiles of wild-type mouse embryonic fibroblasts (MEFs) and those carrying distinct oncogenic lesions in the presence and absence of intact p53. Using semi-quantitative real-time PCR, we examined a panel of 145 miRNAs in wild-type or $p53^{-/-}$ MEFs that over-express Myc, Ras, or E1A plus Ha-RasV12. Notably, miRNA expression was strongly affected by the genetic alterations in MEFs, as unsupervised clustering successfully clustered independent MEF lines according to their genetic lesions. Interestingly, a family of three homologous miRNAs, including mir-*34a, miR-34b* and *miR-34c*, exhibited an expression pattern that precisely correlated with the p53 status, with very little expression detected in all $p53^{-/-}$ MEFs examined. These observations raised the possibility that *miR-34a, b* and *c* might form part of the p53 tumor suppressor network.

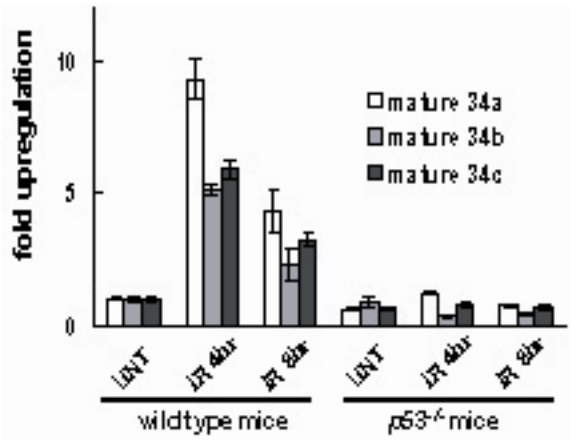

Fig.2 mir-34 family miRNAs are induced by DNA damage in vivo. mir-34a, mir-34b, mir-34c are induced by ionizing irradiation in wildtype mice, but not in p53 deficient mice.

Multiple physiological stresses can, through distinct signaling pathways, induce accumulation of p53 protein and activate p53-mediated transcriptional programs. For example, DNA damage mediates p53 activation mostly through post-translational modification, while oncogenic stress triggers p53 activation through the induction of ARF. miR-34s were induced by ionizing irradiation in a variety of tissues in wild type mice, including spleen, colon, thymus and kidney (Fig2). In contrast, miR-34 induction was not observed in p53-deficient animals. p53-dependent miR-34 up-regulation was also observed in primary MEF cultures when DNA damage was induced by Adriamycin treatment. The amplitude and the kinetics of miR-34 induction closely resemble those of the canonical p53 target, p21, an early p53 response gene that is induced upon DNA damage. In fact, the mir-34 miRNAs are among the most dramatically increased miRNAs induced by adriamycin in human tumor cells

The promoter regions of *miR-34*a and *miR-34b/34c* each contain a palindromic sequence that matches the canonical p53 binding site. These putative p53 binding sites are located within promoter regions that exhibit evolutionary conservation. To verify p53 binding to these putative p53 recognition sites, we carried out chromatin immunoprecipitation (ChIP) with a p53-specific antibody. In wild type MEFs in which p53 activity was induced by DNA damage, regions containing putative p53 binding sites were substantially enriched in p53 immunoprecipitates compared with other regions of the *miR-34* genes or with the same regions in p53-null MEFs. In a genome wide p53 ChIP analysis of human colon cancer cell lines, Chen et al. detected p53 binding to the same sites using; however, these authors did not assign these p53 binding sites to mir-34 promoters. Furthermore, levels of miR-34 correlate with p53 status in tumor and adjacent non-involved tissues from cancer patients. These findings indicate that *miR-34*a and *miR-34b/34c* are direct transcriptional targets of p53 under physiologically relevant conditions (Fig3)

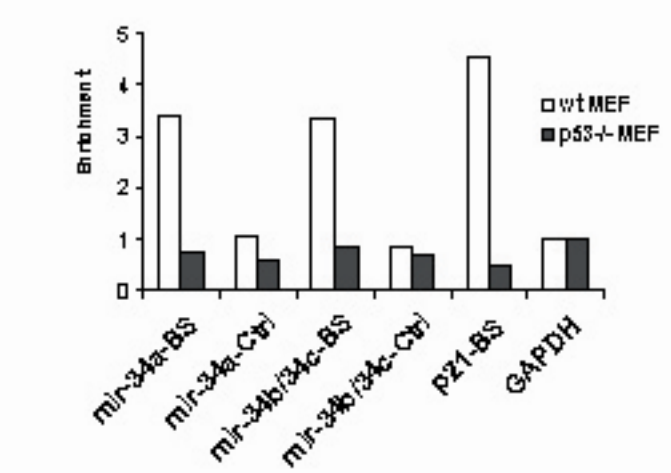

Fig3 mir-34s are direct p53 targets. The promoter region of mir-34a and mir-34b/c are enriched in p53 associated chromatin from wildtype MEFs but not p53-/- MEFs, upon DNA damage.

mir-34s can mediate growth arrest

The outcome of p53 activation is determined by a number of factors, including the cell type, genetic compositions and environmental factors. The two primary end points of p53 pathway activation are apoptosis or growth arrest, either transient (cell cycle arrest) or permanent (senescence). In accord with its acting as a p53 effector, ectopic expression of *miR-34*a or miR-34b/c in IMR90 cells led to a substantial reduction in cell growth. This was attributable to effects on cell proliferation rather than cell death, as the fraction of cells in S-phase decreased, while the fraction of cells in G1 and G2 increased upon *miR-34* expression. The more pronounced impact of *miR-34*b/c expression is most likely due to higher levels of mature miRNAs being achieved with that expression construct. In growth arrested IMR90 cells expressing either *miR-34*a or *miR-34*b/c, we noted distinctive morphological alterations characteristic of cellular senescence (Fig4). Consistent with this observation, more than 60% of cells stained positively for the senescence marker, SA-β-gal at 6 days post selection. Similar observations were made in mouse embryonic fibroblasts, as mir-34 over-expression led to a decrease of S-phase population, and an increase of the G1 and G2 population.

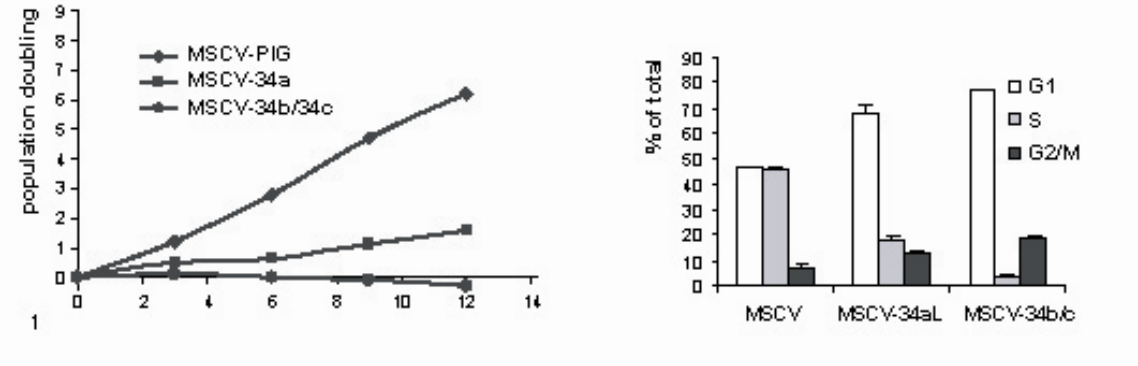

Fig4. mir-34 mediated growth arrest in IMR90 cells. A) Primary fibroblasts IMR90 cells overexpressing mir-34a or mir-34b/c exhibit significant growth arrest. B) IMR90 cells overexpressing mir-34a or mir-34b/c have decreased S phage population and increased G1 and G2/M populations in BrdU analysis.

KEY RESEARCH ACCOMPLISHMENTS

- Identified the functional importance of mir17-92 in the tumorigenesis of breast cancer

- Identified a new miRNA component for the p53 pathway

REPORTABLE OUTCOMES

miRNAs have emerged as integral components of the oncogene and tumor suppressor pathway over the past few years. We have identified novel miRNA components in the key molecular pathways regulating tumorigenesis and are in the process of evaluating the importance of these findings in therapeutical applications.

CONCLUSIONS

miRNAs have emerged over the last two years as an integral component of the oncogenic and tumor suppressor pathways. We have made substantial progress towards understanding the role of miRNAs in breast tumor formation.

REFERENCES

1. L. He, G. J. Hannon, Nat Rev Genet 5, 522 (Jul, 2004).
2. G. A. Calin et al., Proc Natl Acad Sci U S A 99, 15524 (Nov 26, 2002).
3. G. A. Calin et al., Proc Natl Acad Sci U S A 101, 2999 (Mar 2, 2004).
4. S. M. Johnson et al., Cell 120, 635 (Mar 11, 2005).
5. L. He et al., Nature 435, 828 (Jun 9, 2005).
6. J. Lu et al., Nature 435, 834 (Jun 9, 2005).